Mutagenic effect of ethyl-methyl sulphonate on in vitro nodal segments of acid lime cv. PKM-1

Lokesh Bora, RM Vijayakumar, and M Ganga

Abstract
Induced mutagenesis is an essential plant breeding tool which involves the generation of allelic variants of genes that modulate the expression of traits. To minimize the population loss due to excess dose rate of mutagens, it is necessary to optimize the dosage (LD₅₀). In a mutation breeding experiment, LD₅₀ gives an indication of the response of different types of explants of a species to mutagen, so that the right dose(s) for the main field experiment can be fixed to obtain beneficial mutants. In the present investigation, axenic nodal segments of acid lime cv. PKM-1 were subjected to different dosages of chemical mutagen (Ethyl methyl sulphonate) ranging from 10 to 45 mM to find their effect on different growth related traits. The results revealed a gradual and significant reduction in survival of nodal segments, with increase in dose of the mutagens. Probit curve analysis was carried out based on the survival of nodal segments and the LD₅₀ was fixed as 25 mM for chemical mutagenesis. Based on the LD₅₀ the population was maintained for further study.

Keywords: in vitro mutagenesis; ethyl methyl sulphonate; acid lime; LD₅₀

Introduction
Acid lime (Citrus aurantifolia Swingle) belongs to the family Rutaceae and sub family Aurantoideae and is commercially grown in tropical and subtropical regions of India. It is the third important citrus fruit after mandarins and sweet orange. India rank fifth among major lime producing countries in the world. In India, lime occupies an area of 0.25 million hectares with an annual production 2.78 million tonnes and productivity of 10.67 metric tonnes per hectare (Anonymous, 2016) [5]. It is cultivated commercially in Andhra Pradesh, Maharashtra, Gujarat, Tamil Nadu, Karnataka and Himachal Pradesh. Because of the relatively high degree of polyembryony exhibited by this fruit, the seedlings are found true to type when seed propagated and seed propagation is still employed in most of the countries. As a consequence, very few clonal varieties have been selected through selection viz., Chakradhar, Pramalini, Vikram, Tenali, Sai-Sharabati, Kagzi Lime, Mangalipattu, PDKV and PKM-1. Creation of variability is a prerequisite for crop improvement in any plant breeding programme. Spontaneous mutations have played an important role in the improvement of certain characters in some of the fruit crops. Induction of variability using physical mutagen, gamma rays has been in practice for long time by breeders. Tissue culture has a potential for improving the effectiveness of mutation induction in several aspects (Mahadevamma et al., 2012) [10]. In fruit crops, mutagenesis has already been used to introduce useful mutants related to dwarfing, blooming time and fruit ripening period, fruit color, self-compatibility, self-thinning, and resistance to pathogens (Janick and Moore 1996) [13]. Chemical mutagens lead to more specific and predictable mutation, and the procedures are easier to manage without specialized, expensive equipment. In vitro culture techniques like direct (from explants) or indirect (from intervening callus) shoot organogenesis and somatic embryogenesis are valuable tools for conserving important indigenous and improved citrus germplasm by slow growth (medium-term) or cryopreservation (long term) protocols. Induced mutagenesis can be profitably employed as a complementary breeding procedure. Both physical and chemical mutagens have been employed to generate the desired variability. In vitro mutagenesis is a biotechnological technique used in order to alter the agronomical characteristics governed by one or few genes in genotypes of great interest, being considered as a fine adjustment of a variety (Santos et al., 2004) [2]. In vitro mutation induction with the use of gamma rays in stem apex appeared to promote greater viability, when compared with somaclonal variation.
Nowadays, mutation using gamma rays is regularly incorporated in the banana breeding program (Smith et al., 2006 and Mishra et al., 2007). The use of in vitro cultures in mutation breeding of fruit crops advantages over the conventional techniques. Generally, mutants differ in their relative effectiveness and efficiency for the production of mutants in different genotypes (Khalil et al., 2011) [14]. Mutation breeding involves the development of new varieties by generating and utilizing genetic variability through chemical and physical mutagenesis (Oladosu et al., 2016) [15].

In crops in which higher efficiency to produce nucellar polyembryos exist it can be highly useful to create diversity. In perennial vegetatively propagated crops emphasis is laid on the proper way of cutting back the V1M1 and V2M1 shoots in order to stimulate outgrowth of axillary buds, thus promoting formation of stable periclinical chimeras. In any breeding programme, undesirable (mutant) genotypes must be eliminated as early as possible, especially for larger trees. This early screening requires early recognition of the desired types, which in turn requires quick production of stable mutant genotypes in mutation breeding. Somewhere between very young and very old buds, an optimum part of the bud wood can be determined, as was reported by Donini (1976) for cherries, grapes, olives and peaches. Greater emphasis is to be given to the use of combined treatment of physical mutagen and growth regulators. The combined use of this can help the breeder in creating better variability thereby better scope for selection. The use of mutation technique with in vitro propagation over plants to outnumbered vegetatively propagated plants is an effective technique in terms of obtaining variation, quick proliferation of mutants and obtaining mutants and subjecting them to screening for biotic and abiotic stress. Therefore the present study was undertaken to determine the sensitivity of Ethyl Methyl Sulphonate so that the mutants recovered can be used to screen against biotic orabiotic stress.

Materials and Methods

Plant Material

The PKM-1 variety is a selection from Kadayam type of Tirunelveli district of Tamil Nadu. Tree is vigorous and easily multiplied as seedlings and layers. Fruits can be harvested throughout the year.

The study was conducted in year 2015-16. Fruits of acid lime cv. PKM-1 were collected from plants raised in experimental Orchard at Horticultural College & Research Institute, Periyakulam, Tamil Nadu. The in vitro experiment was carried out in the Tissue Culture Laboratory of Horticultural College & Research Institute, TNAU, Coimbatore. Nodal segments of in vitro derived axenic cultures after the germination of seed with a period of one month with uniform size (50 per treatment) were used for chemical treatment using EMS. The nodal segments were soaked in filter sterile (0.22 micron) (SigmaAldrich) (freshly prepared in phosphate buffer at pH 7.0) and EMS solution with different concentrations viz., 10, 20, 25, 30, 40 45 mM for 30-45 minutes. After drying, under laminar air flow and then inoculated in the test tubes, the corrected mortality was calculated based on percentage by using Abbott’s formula given below.

\[
\text{Corrected mortality (\%) = } \frac{M_{\text{observed}} - M_{\text{control}}}{100 - M_{\text{control}}} \times 100
\]

Where,

- $M_{\text{observed}}$: Mortality in treatment,
- $M_{\text{control}}$: Mortality in control

Probit analysis

The shoots growth was studied under in vitro and the lethality was being calculated based on probit analysis by SPSS (Finney, 1971). Fifteen fully expanded leaves were taken and the experiments were repeated once. The data were subjected to statistical analysis using standard deviations of the means, and thereafter to analysis of variance. Mean comparisons were carried out using Duncan’s Multiple Range Test ($a=0.05\%$).

Results and Discussion

The success of mutation breeding depends upon rate of mutation, and the mutation efficiency. This experiment forms the basis for conducting the in vitro mutagenesis experiment in acid lime using EMS. The efficacy of in vitro mutagenesis directly depends on several variables associated with the plant, mutagen and the interactions between explant and mutagen. A total of 50 nodal segments derived through axenic culture were shaken for 30 min and then thoroughly washed with sterile distilled water followed by air drying. The lethality was observed at a period of 15 days, with lower doses treatment out of 50 nodal segments 11 were dead, similarly a number of 16, 23, 28 and 37 plants were dead at 20, 25, 30, 35 mM respectively (Table 1). A linear reduction in survival of inoculated nodal segment was obtained with increase in concentration of mutagen. The LD50 for gamma radiation was fixed based on survival percentage observed after the treatment of mutagen and noticed them after a period 15 day under in vitro condition. The survival percentage showed negative relation correlation with increased dose of mutagen.

The nodal segment showed etiolated shoots, sprouts without lacking chlorophyll, albino and stunted growth behaviour. In vitro techniques offer advantage over in vivo, where under controlled conditions plants are being monitored without any disturbance from outer environmental conditions. EMS induces a biased spectrum of G/C-to-A/T transitions, due to the alkylation at the O6 or N7 position of guanine, which leads to the replacement of cytosine with thymine base pairing. EMS also tends to produce several random point mutations and induces a low level of chromosomal breaks and lethal effects (Greene et al., 2003) [12]. These effects provide a competent survival rate and allow subsequent analyses to be performed for both forward and reverse genetics.

From the proposed experiment the LD50 calculated with the help of probit analysis which was observed at 25mM similar range of result are reported by using rough lemon seedlings (Citrus jambhiri Lush) to observe the effect of mutagens on regeneration and growth of in vitro grown epicotyl segments by Sharma et al. (2013) [18] (Fig. 1). The lower and upper dosage mutants were and the mutants sub-cultured from chemical mutagen were subjected to subsequent generations i.e., M1V1, M2V2 and M3V3 to gain homogeneity as also proposed by (Jain et al., 2011; Roux et al., 2001) [8-9]. Selection and keen observation of variants from the group is very important.

Some minor leaf variations were observed such as leaf shape, leaf length and serration in the or also called as chimeral effect (Albina, Chlorina, Viridis and Xantha). Variations in the morphological and physiological characters can be effectively generated and utilized for meaning full purposes in...
future crop improvement program. Usage of chemical mutagen for Induced mutagenesis is an effective tool to break the limits of variability and to create new alterations in a short period of time period (Kumar and Kumar, 2007) [6]. Under in vitro condition in addition, there is no loss of the mutants, as micropropagules are sub-cultured under sterile conditions (Ling et al., 2008) [3].

Mutation breeding has been employed with varying degrees of success in fruit crops for crop improvement. Mutation breeding combined with tissue culture offered an impetuous and excellent chance for improving the genetic variation of citrus by improving the selection technology and by accelerating the breeding time (Novak et al., 1985) [4]. Differential response to chemical mutagen by citrus was noticed. The morphological mutants based on the visual characters were subjected to generations second and third by means of sub culturing.

Conclusion
The lethal dosage of the experiment was useful to set the mutation experiment and to generate variability. The mutants subjected to subsequent generation may be multiplied and then screen for biotic or abiotic stress under in vitro condition.

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References

Table 1: Effect of mutagen on survival of nodal segments in acid lime cv. PKM-1

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Number of treated</th>
<th>No. of dead</th>
<th>No. of survive</th>
<th>Probability</th>
<th>corrected mortality</th>
<th>LD50</th>
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<tr>
<td>Absolute control</td>
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<td>0.396</td>
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Fig 1: Probit curve for calculation of LD50 in nodal segments of acid lime against ethyl methyl sulphonate.

Fig 2: Survival percentage of nodal segments treated with different concentration of Ethyl methyl sulphonate.

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